Workshop 5.14
Meeting the challenge of the 7th amendment to the EU cosmetics directive (COLIPA Workshop)

Good Science Must be the Key Factor in the Development and Use of Alternative Methods for Safety Assessment of Cosmetics

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Summary
The most striking features of the current EU cosmetic legislation are its animal testing and marketing ban. As the legally imposed timeframe has mainly been introduced by political considerations, the key question remains whether replacement of all in vivo tests is scientifically feasible.
The overview of the current status of 3R alternatives and related deadlines provided by ECVAM were commented by the SCCNFP.
In this paper, repeated dose toxicity testing is discussed to show that innovation is taking place but cannot be governed by political pressure. The opening up of the field of alternatives and the creation of a positive atmosphere towards good science are highly needed.

Keywords: science, alternative methods, safety assessment, cosmetics

Introduction

Both the 7th Amendment of the EU Cosmetic Directive 2003/15/EC (OJ L66, 11/03/2003) and the REACH (Registration, Evaluation and Authorisation of Chemicals) proposal for chemicals (Anon., 2003a) assume that alternatives to animal tests for regulatory purposes are or will be available within the near future.

In particular, the 7th Amendment imposes a prohibition, not only on testing finished cosmetic products and cosmetic ingredients on animals, but also on marketing such products in the EU.

The testing ban on finished products applies from 11 September 2004. So does the testing ban on ingredients, though only when alternative methods become available, i.e. tests that have been officially validated by ECVAM. The maximum cut-off date for the testing ban is 11 March 2009, irrespective of the availability of non-animal alternatives. The marketing ban will be introduced at the latest on the same date, but with the exception of repeated dose toxicity, reproductive toxicity and toxicokinetic testing. For those, the deadline is fixed on 11 March 2013. In the REACH proposal (Anon., 2003a), it is indicated that a scientific objective for the EU is the development and validation of alternative methods and that such methods must be considered as and when they become available. Thus an important difference between both is that in the chemical legislation proposal the 3Rs concept of Russell and Burch (1992) is fully considered, whereas in the cosmetic legislation only replacement was taken up.

The concept of using alternative methods to animal testing was first introduced in the EU legislation by Directive 86/609/EEC (OJ L358, 18/12/1986) on the protection of animals used for experimental and other purposes, which was the immediate reason why ECVAM (the European Centre for the Validation of Alternative Methods) was established in 1992. Alternatives were meant to include all of the 3Rs, being methods to replace, reduce or refine animal tests. Only in the cosmetic legislation was this inscribed and subsequently turned into 1R as a direct consequence of political pressure and excessive lobbying of the Parliament. Thus it is important to notice that the actual deadlines of 2009 and

2013 were not introduced owing to new scientific developments or break-through methodologies becoming available, but rather on account of political considerations.

The crucial question therefore remains whether development of all alternatives needed to guarantee safety for human health is scientifically spoken possible within the limited timeframe foreseen.

Safety evaluation of cosmetics according to the EU cosmetic legislation

In the EU, cosmetics must be safe for human health and the responsibility for this lies with the manufacturer, marketer or first importer into the EU market. The safety assessment of cosmetics takes into account the chemical structure of all ingredients, their toxicological profile and exposure. Toxicological testing of cosmetic ingredients/chemicals is traditionally done using experimental animals, but nowadays preference is given, whenever possible, to alternative methods. Appropriate safety data must be available for all ingredients, regardless of the tonnage of marketing within the EU, in order to permit safety evaluation (= risk assessment) of the finished cosmetic product (Pauwels and Rogiers, 2004).

In Europe, two distinct channels are functional (fig. 1) (Anon., 2003b). Basically, the substances present in Annexes II, III, IV, VI and VII of the Cosmetic Directive fall under the responsibility of the SCCP (Scientific Committee on Consumer Products, the former SCCNFP) (left part of fig. 1).

The right part of figure 1 contains all ingredients of cosmetic products other than those present in the Annexes. They fall under the responsibility of the manufacturer through the safety assessor. In fact, a specific data package must be kept readily accessible to the EU Member States' Competent Authorities. This is called a Technical Information File (TIF) or a Product Information Requirement (PIR) (Pauwels and Rogiers, 2004).

The set of tests to be considered for the determination of the toxic potential of the ingredients present in the Annexes is represented in table 1 (Anon., 2003b).

For the ingredients not present in the annexes, usually acute toxicity, local toxicity, dermal absorption, repeated dose toxicity and mutagenicity are considered to be the minimal base set requirements (Anon., 2003b). According to Directive 92/32/EEC (OJ L54, 05/06/1192) on the classification, packaging and labelling of dangerous substances, the toxicological requirements for substances produced/EU imported at levels between 100 kg and 1 tonne per year consist of the minimum set

Tab. 1: General toxicological requirements for cosmetic ingredients, present in the Annexes of the Cosmetic Directive.

Acute toxicity (if available)
Irritation and corrosivity
Skin sensitisation
Dermal absorption
Repeated dose toxicity
Mutagenicity/genotoxicity
Carcinogenicity
Reproductive toxicity
Toxicokinetics
Photo-induced toxicity
Human data

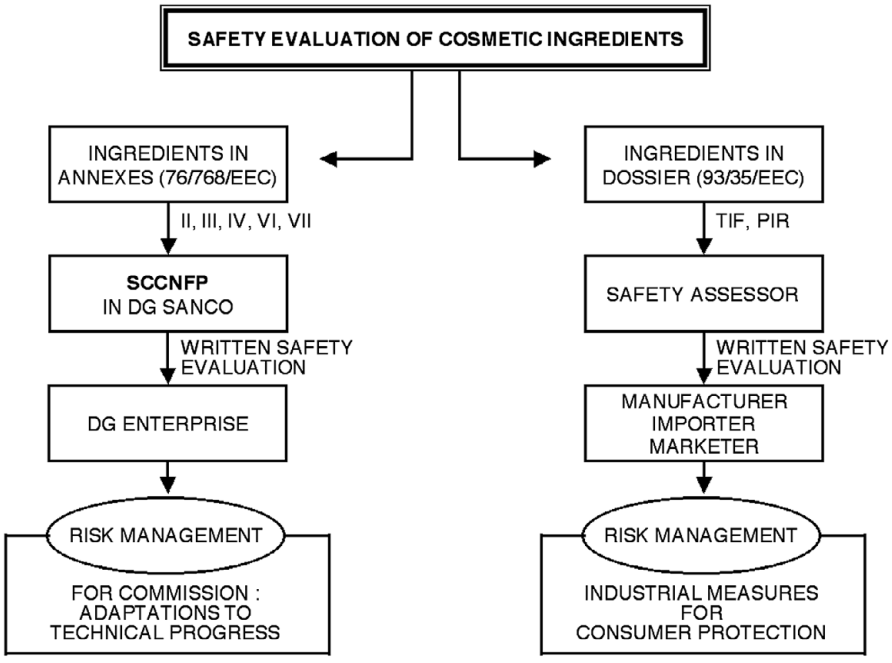

Fig. 1: Two distinct channels for safety assessment of cosmetic ingredients are functioning in the EU (Anonymous, 2003b).

just mentioned, but without dermal absorption and repeated dose toxicity. Thus, not all of the test results required for the assessment of cosmetic ingredients are automatically generated through the chemicals' legislative procedures. Only when higher amounts of substances are involved, a more extensive list of toxicological requirements is established.

Alternatives to animal testing

Alternatives do not yet exist for all toxicological tests required to guarantee the safety of cosmetic ingredients for human health (Anon., 2003b). A recent overview of the current status and future prospects of alternatives has been prepared by ECVAM (Anon., 2004a) and was recently published (Eskes and Zuang, 2005). It provides a good overview of all 3R alternatives that exist or are under development.

The original ECVAM document was commented upon by the former SCCNFP (Anon., 2004b) and the following clear message was given to the Commission:

-"total abolishment of animal tests within 10 years is not feasible from an objective scientific point of view. Even the alternative strategies discussed in the document, which are estimated to take more than 10 years for further development, still include an animal test in the finalities."

-"In many cases (especially for systemic toxicity), all the gaps are not identified."

-"Following fields are missing throughout the document:

- absorption through mucosa
- respiratory sensitisation
- in the repeated dose toxicity and developmental toxicity sections, two of the "3R" (refinement and reduction) have been neglected
- upcoming alternative technologies, such as the use of stem cells, genomics/proteomics… and tissues and cells derived from transgenic rats and in particular transgenic mice, have also been overlooked
- some areas of reproductive/ developmental toxicity have not been covered…"

Also, the expert members of the former CSTEE (Scientific Committee on Toxicity, Ecotoxicity and the Environment) came to the conclusion that, for the foreseeable future, the use of live animals in toxicity testing remains essential in order to perform reliable risk assessments (Anon., 2004c).

Thus two expert committees of the Commission feel that, scientifically spoken, the development of alternatives has not yet reached the stage that one would tend to believe from the context of the current cosmetics legislation (2003/15/EEC, OJ L66, 11/03/2003) and coming chemicals legislation (Anon., 2003b). The approach of pushing legislation without having the necessary scientific knowledge appears to be quite threatening for the safety of cosmetics and their free circulation within and outside the EU market.

In order to support the replacement of *in vivo* by *in vitro* methods, research projects have been initiated within the Sixth Framework Research Programme of the EU, in particular for some of the fields where alternatives are still lacking at the moment (e.g. ReProTect, AcuteTox, Predictomics, Sens-it-iv and others). It must be emphasised, however, that these projects only started very recently and that the first results can only be expected around 2010.

Moreover, validation is a time-consuming activity that can only start after proper development of a relevant method and its prevalidation (Fentem and Balls, 1997). From the past, we know that it may take up to 8 years to have a method ready and implemented into the legislation. Finally, one must recognise that for certain endpoints, such as repeated dose toxicity testing, no acceptable proposals for *in vitro* development exist at this moment (Prieto et al., 2005).

One can argue that science advances quickly and proposals will emerge within the near future. Unfortunately, reality has shown differently. A literature search over the past 6 years (1998-2003) was carried out in Germany using standard databases including Chemical Abstracts, Medline, Embase, Biosis, SciSearch, Derwent Drug Pat., SCRIP Ring doc-ZEBET, ICCVAM (The Interagency Coordinating Committee on the Validation of Alternative Methods), and Monographs (Garthoff, 2003, 2005). It appeared that, of the apparently impressive number of articles published on the topic of 3R alternatives, most were reviews and only a limited number contained original research results (22 articles over 6 years).

Furthermore, most articles were not published in top scientific journals, which also handicapped their impact.

The case of repeated dose toxicity testing: Subacute and subchronic toxicity

Of relevance for cosmetic products and their ingredients are oral, dermal and inhalation tests, for which several OECD and EU-accepted *in vivo* tests are available today.

The currently indispensable oral repeated dose toxicity testing is still carried out in animals (rats and/or dogs) and, as mentioned before, to date no scientifically acceptable alternative methods exist (Eskes and Zuang, 2005). The current *in vivo* tests provide a so-called NOAEL (no observable adverse effect level) for the ingredient under consideration, a value that gives an estimation of the dosage that causes no adverse effects in the animal after repeated exposure (28 days or 90 days) (Anon., 2003b). This NOAEL is used to calculate the margin of safety (MoS) or uncertainty factor of the ingredient. The systemic exposure (SED) is taken into consideration by the following equation MoS= NOAEL/SED. According to the WHO, the calculated MoS for a given ingredient must be at least 100 in order to consider the ingredient to be safe for human use (Anon., 2003b). Thus the determination of a realistic NOAEL value has key importance in risk assessment.

When considering the replacement of the *in vivo* repeated dose toxicity test, it is enlightening to have a look at the endpoints of such a study in order to understand the complexity of its replacement. As reflected in tables 2 to 7, a large set of measurements and findings are involved. The total number of observations and measurements per test substance and per concentration can easily amount to more than a hundred.

If one aims to replace these complex *in vivo* tests by alternatives, one has to develop a testing strategy that provides the same level of knowledge as obtained *in vivo*. This implies an integrated approach, where *in silico* methodology needs to be combined with a battery of *in vitro* tests, and probably also refined *in vivo* tests, in order to include all relevant toxicological endpoints of different types of organs.

One can imagine that the physico-chemical and biological properties, the ADME (absorption, distribution, metabolism, excretion) and ADMET (ADME under toxicological conditions) data of molecules and classes of molecules (read-across strategy) can contribute (Coecke et al., 2005). Thus SAR and QSAR (quantitative structure activity relationship) and mechanism-based PK/PD (pharmaco-kinetic/pharmaco-dynamic models) analysis (Prieto et al., 2005) data certainly play a role in integrated testing, but their importance should not be overestimated either. They are mainly important as a first step for priority setting and further decision-making.

A whole range of *in vitro* tests such as cultures of primary cells and a variety of cell lines can provide important toxicological information on organs of different species (including man), in particular when these are combined not only with traditional

Tab. 2: Overview of the simultaneous estimation of different measurements and findings in subacute and subchronic toxicity testing, part "clinical observations"

clinical observations	- daily for overt signs and mortality
histopathology	- weekly for :
haematology	- skin and fur condition
body weight	- eyes
food consumption	- mucosa
organ weight	- respiratory function
clinical biochemical parameters	- circulatory function
urinanalysis	- nervous system function

Tab. 3: Overview of the simultaneous estimation of different measurements and findings in subacute and subchronic toxicity testing, part "histopathology"

clinical observations	- brain	- heart
histopathology	- spinal cord	- trachea & lungs
haematology	- pituitary	- aorta
body weight	- (para)thyroid	- gonads
food consumption	- thymus	- uterus
organ weight	- oesophagus	- accessory sex organs
clinical biochemical parameters	- salivary glands	- ♀ mammary gland
urinanalysis	- stomach	- prostate
	- small intestines	- urinary bladder
	- large intestines	- gal bladder
	- liver	- lymph nodes
	- pancreas	- peripheral nerve
	- kidneys	- bone marrow
	- adrenals	- skin
	- spleen	- eyes

Tab. 4: Overview of the simultaneous estimation of different measurements and findings in subacute and subchronic toxicity testing, part "haematology"

clinical observations	- haemoglobin concentration
histopathology	- erythrocyte count
haematology	- packed red blood cell volume (PCV)
body weight	- leukocyte count
food consumption	- platelet count
organ weight	- reticulocyte count
clinical biochemical parameters	- activated partial thromboplastin time
urinanalysis	

Tab. 5: Overview of the simultaneous estimation of different measurements and findings in subacute and subchronic toxicity testing, parts "body weight", "organ weight" and "food consumption"

clinical observations		
histopathology		
haematology		
body weight	- weekly observation	
food consumption	- 3d or 6d intervals (+ water consumption)	
organ weight	At necropsy	Relative weights
clinical biochemical parameters	- brain	- per body weight
urinanalysis	- liver	- per brain weight
	- kidneys	
	- lungs	
	- heart	
	- spleen	
	- thymus	
	- adrenal glands	
	- gonads	

Tab. 6: Overview of the simultaneous estimation of different measurements and findings in subacute and subchronic toxicity testing, part "clinical biochemical parameters"

clinical observations	- glucose
histopathology	- urea nitrogen
haematology	- creatinine
body weight	- total protein, albumin and globulin
food consumption	- bilirubin
organ weight	- phosphorus
clinical biochemical parameters	- Ca^{2+}, Na^{+}, K^{+}, Cl^{-}, HCO_3^{-}
urinanalysis	- aspartate aminotransferase
	- alanine aminotransferase
	- creatine kinase
	- sorbitol dehydrogenase
	- γ-glutamyl transferase
	- alkaline phosphatase
	- glutamate dehydrogenase
	- ornithine carbamyltransferase

Tab. 7: Overview of the simultaneous estimation of different measurements and findings in subacute and subchronic toxicity testing, part "urinanalysis"

clinical observations	- volume
histopathology	- appearance
body weight	- microscopy
haematology	- pH
food consumption	- osmolality
organ weight	- phosphorus
clinical biochemical parameters	- protein content
urinanalysis	- occult blood
	- glucose
	- ketones
	- bilirubin and urobilinogen
	- creatinine
	- α_{2u}-globulin
	- N-acetyl-β-D-glucoaminidase

technology but in particular with new "–omics" technology (Elaut et al., 2002). Indeed, a series of *in* vitro liver, kidney central nervous system, pulmonary, haematopoietic system preparations do exist (Prieto et al., 2005). Of these, liver cell cultures and, in particular, hepatocyte cultures, have been studied very extensively, but even these have not yet been validated or taken up into legislation (Coecke et al., 1999). Hepatocytes in culture, like other primary cells in general, dedifferentiate and loose their specific properties, making them less than ideal for long-term tests (Rogiers and Vercruysse, 1993; LeCluyse, 2001; Elaut et al., 2005).

Only recently an innovative technology was proposed to counteract dedifferentiation of primary cells in culture by inhibiting cell proliferation (Rogiers et al., 2004). The principle is based on the addition of histone deacetylase inhibitors to the perfusion solution during the isolation procedure of the cells and during their culture. These molecules are known as experimental anticancer drugs (Vanhaecke et al., 2004a). They interact with the acetylation/deacetylation process of histones in the cell nucleus (Lopez-Rodas et al., 1993; Magnaghi-Jaulin et al., 2000; Gregory et al., 2001). Hyperacetylated histones are formed, which result in DNA molecules becoming more accessible for transcription factors. Effects on the stabilisation of primary hepatocytes exposed to histone deacetylase inhibitors are available. Interesting results have been measured on cell cycle parameters (Papeleu et al., 2003, 2005, 2006), gap junctional intercellular communication (Vinken et al., 2006), apoptosis (Vanhaecke et al., 2004b), xenobiotic biotransformation (Henkens et al., 2005), and stem cell technology (Snykers et al., 2006). This new methodology is believed to be equally applicable to other primary cells (Rogiers et al., 2004) and research is ongoing.

Under the assumption that this innovative technology will result in the production of stable, functional primary cells of different organs, one still has to perform their full characterisation. Relevant markers and toxicological endpoints have to be developed for each of the toxicological targets. Furthermore, as it is known that *in vivo* all organs and tissues function in a coordinated and integrated way, it becomes crucial to also develop *in vitro* interrelationships between the different culture systems. Until now, the latter has been completely ignored.

What this example clearly shows is that innovation indeed takes place and opens perspectives, even in the context of a replacment strategy for repeated dose toxicity testing, but also that it follows its own rhythm and not that imposed by fixed timeframes.

Conclusions

The basic message is that scientific development continues as long as scientists are offered a positive environment for their work in terms of adequate funding, an up-to-date technological environment and a perspective for a decent carrier.

Innovation and break-through results, however, cannot be forced but are the normal outcome of continuous efforts and building up of knowledge by dedicated scientists. Thus scientific work follows its own rules and it seems absurd to try to impose legislative time deadlines as a way of putting pressure on the scientific community.

Efforts to speed up the outcome of results, however, can be obtained by a positive attitude towards opening up the field of alternatives by

Reorientation

(i) to attract top scientists to the field of alternatives,

(ii) to attract scientists of areas of science not yet involved in alternatives;

(iii) to stop focusing on cosmetics.

Rejuvenation

(iv) to provide more and better research opportunities for young researchers;

(v) to involve more young scientists in decision-making and strategy-building.

Requalification

(vi) to better coordinate and prioritise research projects on alternatives;

(vii) to stimulate scientists to invest in quality and to publish in scientific journals with a high impact factor.

Rather than political lobbying for non-realistic legislative deadlines and blocking the whole field, efforts could better be combined to stimulate a positive environment for good science.

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